

COMMENTARY

That zincing feeling: the effects of EDTA on the behaviour of zinc-binding transcriptional regulators

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Zinc-binding proteins account for nearly half of the transcription regulatory proteins in the human genome and are the most abundant class of proteins in the human proteome. The zinc-binding transcriptional regulatory proteins utilize Zn^{2+} to fold structural domains that participate in intermolecular interactions. A study by Matt et al. in this issue of the *Biochemical Journal* has examined the transcription factor binding properties of the zinc-binding module C/H1 (cysteine/histidine-rich region 1) found in the transcriptional co-activator proteins CBP (CREB-binding protein) and p300. Their studies revealed that EDTA treatment of native C/H1 leads to irreversible denaturation and aggregation. Of particular concern is their finding that unfolded C/H1 parti-

cipates in non-specific protein–protein interactions. The implications of these results are significant. EDTA is a very potent zinc-chelating agent that is used ubiquitously in protein interaction studies and in molecular biology in general. The potentially detrimental effects of EDTA on the structure and interactions of zinc-binding proteins should be taken into account in the interpretation of a sizeable number of published studies and must be considered in future experiments.

Key words: CREB-binding protein (CBP), p300, chelation, denaturation, EDTA, transcription factor, zinc-binding domain, zinc finger.

Zinc-binding proteins represent the largest and most complex gene superfamily in metazoans and comprise the most common class of transcription factors. Close to one thousand Cys2His2-type zinc-finger transcription factors are encoded by the human genome, representing nearly half of the hypothetical genes involved in the regulation of gene expression [1]. These zinc-binding proteins include co-activators, chromatin-modifying and -remodelling enzymes, a large number of DNA-binding transcription factors, members of the general transcription machinery and multisubunit RNA polymerases. The importance of zinc in gene expression cannot be overstated.

The extensively homologous cellular co-activators CBP (CREB-binding protein) and p300 (CBP/p300) are prominent members of the zinc-binding superfamily. These very large cellular co-activators appear to play a role in the integration of gene expression in all metazoans. Over 1500 papers characterizing the physical and functional properties of CBP/p300 have been published during the last decade. Based on a large body of protein–protein interaction data, CBP/p300 have been suggested to carry out their co-activator function via direct binding to a vast number of structurally unrelated transcription regulators. The protein–protein interactions occur through several conserved domains within CBP/p300. Prominent among these are cysteine/histidine-rich regions 1 and 3 (C/H1 and C/H3), each of which contains cysteine/histidine-rich sequences that co-ordinate three Zn^{2+} atoms. These well-characterized domains form Zn^{2+} -dependent helical fold structures that stabilize intermolecular interactions and mediate the recruitment of CBP/p300 to promoter-bound transcription factors [2–4]. A third zinc-binding region of CBP/p300, called C/H2, comprises a highly conserved PHD (plant homeodomain)-type zinc finger that is critical for the enzymic activity of the histone acetyltransferase domain.

In this issue of *Biochemical Journal*, Matt et al. [5] examined the structure and protein–protein interaction properties of the CBP/

p300 C/H1 domain in the presence and absence of Zn^{2+} . The authors [5] found that incubation of native C/H1 with EDTA produced CD spectra with significantly decreased α -helical content, consistent with protein unfolding in the absence of Zn^{2+} that is typical of small zinc-binding motifs [6]. The addition of excess Zn^{2+} did not restore C/H1 to its native structure, suggesting that C/H1 denaturation by EDTA was irreversible. The authors then used GST (glutathione S-transferase) pull-down assays to show that native C/H1 bound to one of its well-characterized binding partners, HIF1 α (hypoxia-inducible factor 1 α). Notably, this interaction was abolished when EDTA was included in the binding reaction. These results are consistent with previous reports showing that the structural integrity of C/H1, and its ability to interact with HIF1 α , is dependent upon proper folding in the presence of Zn^{2+} [2,4].

Of greater significance is the observation by Matt et al. [5] that HDM2, the human homologue of the well-characterized C/H1-binding partner MDM2 (mouse double minute protein), does not bind to native C/H1. Instead, HDM2 was found to non-specifically interact with the unstructured form of C/H1 present under EDTA-induced denaturing conditions. The interaction between C/H1 and MDM2 has been reported by several laboratories, and Matt et al. [5] point out that all of these previous experiments were performed in the presence of EDTA (see [5] for specific references). The results presented in this issue of *Biochemical Journal* suggest that non-specific binding of MDM2 to unfolded C/H1 was responsible for the observed interactions.

The implications of this work are extraordinary. The C/H1–MDM2 interaction represents just one of many reported interactions with the zinc-binding C/H1 and C/H3 domains of CBP/p300. If the C/H1–MDM2 interaction is non-specific, then there are likely many additional transcription factor interactions with CBP/p300 that need to be re-examined. Furthermore, given the prominence of zinc-binding proteins in the regulation of gene

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expression, questions regarding protein–protein interactions with the zinc-binding modules of CBP/p300 are likely just the tip of the iceberg. A cursory review of approx. 100 papers from the recent literature revealed that over 80 % of the reported protein–protein and protein–DNA interactions with known zinc-binding transcription factors were studied using EDTA-containing reaction buffers. These include GST pull-down, electrophoretic mobility-shift and co-immunoprecipitation assays. The work presented by the Matt et al. [5] leads to the unavoidable conclusion that at least some of these published experiments were performed with partially or fully unfolded proteins.

The prevalence of EDTA in protein–protein interaction assays is not surprising, because its use has become nearly ubiquitous in the field of molecular biology. EDTA has been used for many decades as an inhibitor of metalloproteases and DNases and as a scavenger of trace heavy metals. EDTA concentrations of 0.1–5 mM are common in buffers used for protein purification, DNA and nuclear extract preparations, and a wide variety of protein–protein and protein–DNA interaction assays. EDTA is also frequently used in running buffers (e.g. Tris/borate/EDTA) for ‘native’ electrophoretic mobility-shift assays. Additionally, EDTA may be inadvertently introduced with some component of a reaction mixture, resulting in a low concentration of EDTA that may still be sufficient to chelate all the Zn^{2+} in the solution and disrupt the structure of zinc-binding motifs.

The zinc-binding motifs found in transcription factors have dissociation constants in the range of 10^{-9} – 10^{-11} M, whereas EDTA binds zinc extremely tightly with a K_d of approx. 10^{-16} M [7]. As a result, EDTA will very effectively compete the zinc away from these proteins. The high affinity of EDTA for these metals is the result of a very fast and essentially diffusion-limited association rate coupled with a very slow dissociation rate. Furthermore, EDTA binds Zn^{2+} much more tightly than other common divalent metals such as Mg^{2+} (K_d , approx. 10^{-9} M) and Ca^{2+} (K_d , approx. 10^{-11} M). EDTA will therefore selectively deplete a solution of free Zn^{2+} , even in the presence of high concentrations of these other divalent cations. Unfortunately, there is not much literature on the kinetics of Zn^{2+} binding and release to and from zinc-finger-type proteins. We can estimate the rates using data from EF-hand Ca^{2+} -binding proteins that typically have association rates (k_{on}) on the order of 10^7 – $10^8 \text{ M}^{-1} \cdot \text{s}^{-1}$ [8]. If we assume the ‘on’ rates for Zn^{2+} binding to zinc-finger-type proteins are comparable, then their ‘off’ rates are in the order of 0.1 – 10^{-4} s^{-1} based on the relationship $K_d = k_{\text{off}}/k_{\text{on}}$. In practical terms, this means that even a few minutes of exposure to EDTA can result in significant stripping of Zn^{2+} from many proteins. We should point out that the Zn^{2+} dissociation rates can vary greatly among proteins and the formation of specific protein–protein complexes may slow dissociation by stabilizing the metal-bound structure, as seen with Ca^{2+} -binding proteins [8]. Many larger proteins fold to create non-structural zinc-binding sites where the removal of metal may affect activity, but leave the structure and protein–protein interactions intact. However, the fastest off rates and greatest experimental complications will probably come from small Zn^{2+} -coordinating domains, such as C/H1 and C/H2, where the bound

metal is required to stabilize the native conformation of the domain.

Another very important observation is that some zinc-binding domains may not refold into their true native structure after the removal of EDTA and addition of Zn^{2+} . For example, C/H1 is irreversibly denatured [4,5], whereas Sp1 [9] and certain nuclear hormone receptors [6] can be refolded following the removal of EDTA and the addition of micromolar concentrations of Zn^{2+} . It is therefore important to appreciate that the use of EDTA at any point in a preparation or assay may result in significant Zn^{2+} chelation that leads to domain denaturation and potentially erroneous results. One must also be aware of the fact that the Zn^{2+} -binding properties of many proteins are not known.

The effects of Zn^{2+} and EDTA on the C/H1–MDM2 interaction indicated by Matt et al. [5] represent a potentially serious and complicated issue that may have broad implications for the interpretation of data obtained using common molecular biological techniques. It is paradoxical that the inclusion of EDTA can lead to both false-negative and false-positive results. False-negative results arise when zinc-binding domains are unfolded in the presence of EDTA and thus are unable to interact with their legitimate binding partners. False-positive results arise when the zinc-binding protein unfolds in the presence of EDTA and subsequently interacts non-specifically with other proteins. Given the prevalence of zinc-binding motifs in transcription regulatory proteins, one would be wise to empirically determine the effect of EDTA and Zn^{2+} on molecular interactions observed in gene expression studies *in vitro*.

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